Renewal Assessment Report

Lecanicillium muscarium Ve6

Volume 3MA – B.5 Analytical methods

January 2018

Rapporteur Member State: The Netherlands

Co-Rapporteur Member State: France

Version history

When	What
January 2018	Initial RAR

Table of contents

B Summary of the data and information

B.5 Analytical methods

Note to reader:
Information from the original DAR and/or addenda to the DAR is highlighted grey.

B.5.1 Methods for the analysis of the micro-organism as manufactured

B.5.1.1 Methods for the identification of the micro-organism

Information from the original DAR

DAR addendum (June 2009)

Reporting table 1(1)

The notifier stated that, as far as they understand, the new species *L. muscarium* is conspecific with the old strain *V. lecanii* Ve6. The strain identifier "Ve6" was an internal number used at Tate & Lyle, and is no longer used. The notifier suggests to use as the strain identifier "19-79" in stead of Ve6, since this refers to the sample in the IMI collection and to the original strain that was developed by Dr. R. Hall and later by Tate & Lyle, and still is in the product MYCOTAL. This reference is also mentioned by Zare and Gams (2001).

Data gap: 1.1 (see reporting table 1(2))

Method of analysis to unequivocally identify this strain should be provided.

Comment from notifier:

The methods genetic fingerprinting (RELP), ITS and mtDNA were used for identification and distinguishing the number of Verticillium isolates, particularly Vertalec and MYCOTAL (Typas, 2002a; Appendix IIM 1.3.3-06;Appendix IIM 1.3.3-07). These data were generated in the EU RAFBCA project (QLK1-CT-2001-01391). In the dossier, Koppert refers to the preliminary and therefore unpublished results of Typas (2002a) as a part of above project. In 2008 Typas's laboratory published their methods and results from RAFBCA project on classification and identification of more than 60 isolates including Vertalec and MYCOTAL with information of their accession numbers in the public GenBank (Kouvelis et al., 2008).

MYCOTAL strain *V. lecanii* Ve6/*L. muscarium* 19-79 has ITS and NMS accession numbers EF512971 and EF513028, respectively. Vertalec strain *V. lecanii/L. longisporum* Ve2 has ITA and NMS accession numbers EF512970 and EF513029, respectively.

Typas (2002a, unpublished, later becomes published as Kouvelis et al, 2008)

An analysis of Vertalec and MYCOTAL isolates by genetic fingerprinting

In the study by Typas, the MYCOTAL isolate (=strain Ve6/19-79) was examined together with 29 more *Verticillium lecanii* isolates, collected from different hosts and geographic locations. The tools used were as follows:

a) RFLPs and DNA hybridisations

b) PCR of the nuclear rRNA gene complex

c) PCR of mtDNA

Typas (2002a) concluded that:

- MYCOTAL can be differentiated from all other *V. lecanii* isolates (each one) by restricting total DNA with tetra-cutter enzymes and comparing their RFLPs, in combination with the mtDNA PCR amplicon restriction patters.

- IGS sequences and intergenic mtDNAsequences provide an indisputable “genetic fingerprinting identity” for *V. lecanii* isolates.

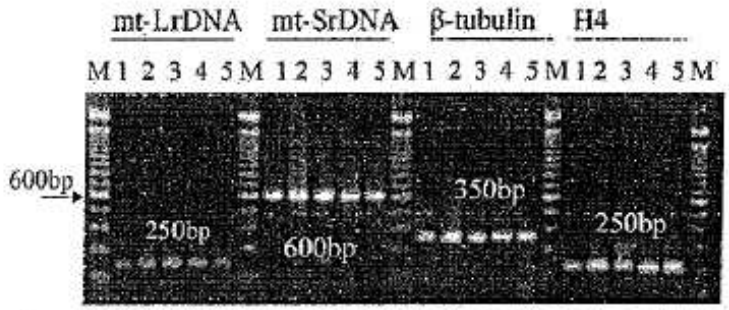


Fig. 2. Agarose gel electrophoresis of PCR products of mt-LrDNA (ML1/2), mt-SrDNA (MS1/2), β-tubulin gene (Bt-2) and H4 gene (H4-1) regions amplified from *V. lecanii* isolates. See Section 2 for abbreviations and primer sets in parentheses. Lane M is 100-bp ladder molecular weight marker. Isolates in gel lanes are as follows: 1, MAFF235142; 2, Mycotal; 3, Vertalec; 4, MAFF238426; 5, ATCC58909.

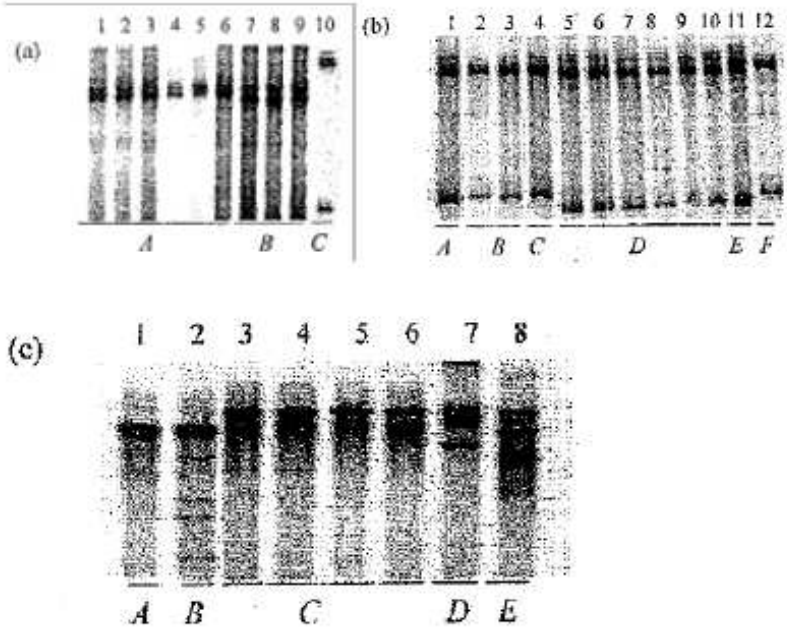


Fig. 3. PCR-SSCP profiles of *V. lecanii* isolates produced by three variable genomic regions were separated electrophoretically on acrylamide gels followed by silver staining. Regions amplified by PCR were mt-LrDNA (a), β-tubulin (b) and H4 (c). SSCP type is indicated by letters in italics at the bottom of the figure. (a) Lanes: 1, MAFF235142; 2, MAFF235700; 3, MAFF425193; 4, MAFF238425; 5, ATCC22611; 6, Mycotal; 7, Vertalec; 8, MAFF235701; 9, ATCC46578; 10, ATCC58712. (b) Lanes: 1, MAFF235679; 2, MAFF235698; 3, MAFF425193; 4, Mycotal; 5 Vertalec; 6, MAFF235690; 7, MAFF235696; 8, MAFF235699; 9, MAFF235701; 10, ATCC46578; 11, ATCC58712; 12, ATCC60540. (c) Lanes: 1, MAFF235140; 2, MAFF235686; 3, MAFF235696; 4, Mycotal; 5, ATCC22612; 6, ATCC46578; 7, Vertalec; 8. ATCC58712

Note RMS: It is indicated that mtDNA were used for identification, please detail the method used on mt DNA. For clearity reason, the fragments length should have been detailed in figure 2 and specifically for figure 3. The difference between the strain V. lecanii Ve6/ L. muscarium and others strains is not clear.

More details on the methods used for the identification have to be provided (restriction enzymes used, detail of the specific sequence and of the primers). Furthermore, a phylogenetic tree using a specific DNA sequence has to be provided and a comparison with the closed stains has to be performed using a molecular method. Otherwise the results provided are not sufficient to identify V. lecanii Ve6/ L. muscarium at strain level.

B.5.1.2 Methods for providing information on possible variability of seed stock/active micro-organism

Information from the original DAR

According to the notifier major changes in characteristics of *Verticillium lecanii* Ve6 will result in a change in germinability and effectiveness in bio-assay, hence a change in characteristics can be detected in the viable spore content test and the qualitative bio-assay.

The amount of spores in formulated product is determined using a Bürker-Turk counting chamber.

For the determination of the amount of viable spores it is necessary to determine the germination-percentage of technical spore powder or product. Dilutions of the suspension are plated on growth medium (SDA or water agar). The efficacy of the batches is estimated by using a bioassay, in which leaf-discs and insects are treated and kept at environmental conditions comparable to those in protected crops.

New data 2016

See Volume 4.

B.5.1.3 Methods to differentiate a mutant of the micro-organism from the parent wild strain

Information from the original DAR

Not applicable. *Verticillium lecanii* Ve6 is a naturally occurring strain, which has not been artificially mutated or genetically modified.

B.5.1.4 Methods for the establishment of purity of seed stock from which batches are produced and methods to control that purity

Information from the original DAR

The seed stock is stored in an appropriate manner. Each new batch is cultured under sterile conditions, and is kept no longer than 3 months in at 2-5°C.

B.5.1.5 Methods to determine the content of the micro-organism in the manufactured material used for the production of formulated products and methods to show that contaminating micro-organisms are controlled to an acceptable level

Information from the original DAR

The notifier tests every batch of technical spore powder and final product (by an independent laboratory). If the criteria are not met, the batch is not used or further developed.

The contaminants have to be less than 500.000 colony forming units (CFU) per gram of technical spore powder and per gram of the final formulated product.

DAR addendum October 2009

Validation for method used to quantify viable spores and for the whitefly bioassay in the stability studies is required.

Specificity, linearity, accuracy and repeatability of method to determine viable spore content Verticillium lecanii strain Ve6.

Specificity

Spores are counted at a 1000× magnification to determine the viable spore content. At this magnification spores can be identified on their morphology. Spores of different fungi will be recognised as such and will not be counted.

Linearity

The viable spore content of spore powder or formulated product is determined by counting spores in a counting chamber according to the method of Bürker-Türk and by counting the number of germinated spores out of 100 spores at 1000× magnification. These are direct observations. Therefore determination of linearity of these methods is not relevant.

Accuracy

Spore counting in a counting chamber according to the method of Bürker-Türk is a generally accepted accurate method and is generally used in science to count spores. The data obtained from quality control tests of 4 batches are presented in **Table 1**. The standard errors of the spore count and germination average are low, demonstrating the accuracy of the data.

Table 1 Spore count in counting chamber and % germination of 4 batches of *Verticillium lecanii* strain Ve6

	Batch 98M397		Batch 98M399	
	spore count	germination	spore count	germination
Average				
Standard error				

	Batch 97M401		Batch 97M403	
	spore count	germination	spore count	germination
Average				
Standard error				

Precision

The viable spore contents of batches 97MYC401, 97MYC403 and 98MYC399 have also been determined by the Fungal Biodiversity Centre (CBS) in July 2003. These counts are compared with the counts by Koppert on 24 June 2003 (98MYC399), 23 July 2003 (97MYC401) and 06 August (97MYC403) in Figure 1. Differences between test results of Koppert B.V and CBS varied from 10 to 23%.

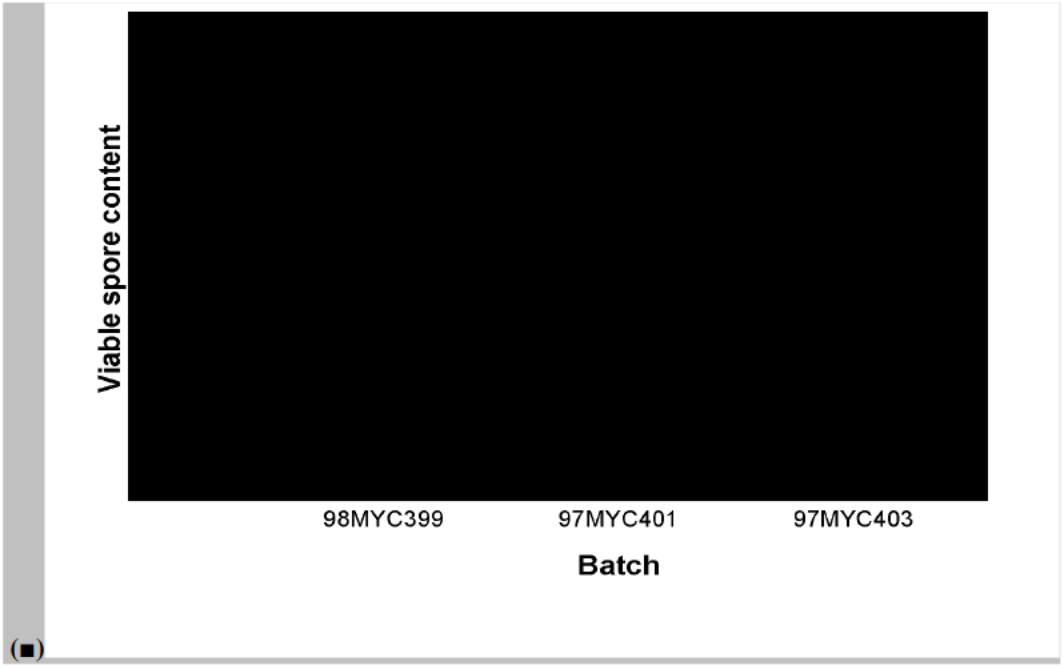


Figure 1 Viable spore count by Koppert BV (□) and CBS

Specificity, linearity, accuracy and repeatability of method to determine the bioassay of *Verticillium lecanii* strain Ve6.

Specificity

The test organisms in the bioassay are whitefly and thrips. Experience over the years demonstrate that *Verticillium lecanii* strain Ve6 causes a mortality of 80 - 100% under the conditions set for this bioassay. Product samples containing abnormal species or strains will result in different mortality rates.

Linearity

The bioassay used to determine the efficacy is based on a standard concentration of product (1 g product/L). The testing of other concentrations is not relevant and linearity of the method has therefore not been determined.

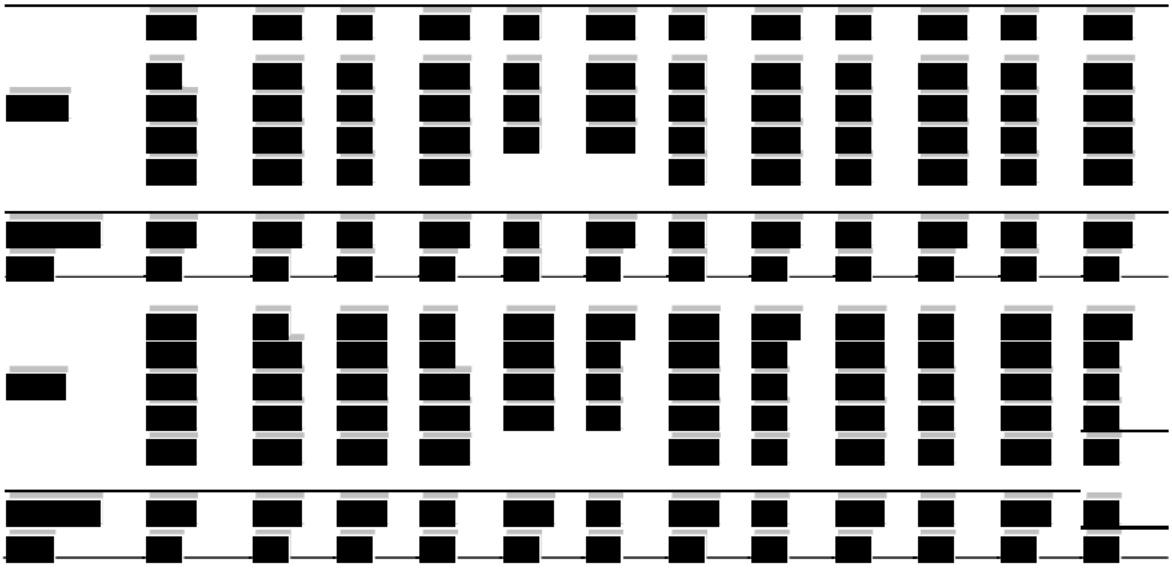
Accuracy

The results of 6 bioassays carried out independently with MYCOTAL from 5 batches have been presented in **Table 1**. Four till five Petri dishes were used per bioassay. The standard errors of the average living, dead (+/- mycelium) and hatched pupae per bioassay are low demonstrating the accuracy of the data.

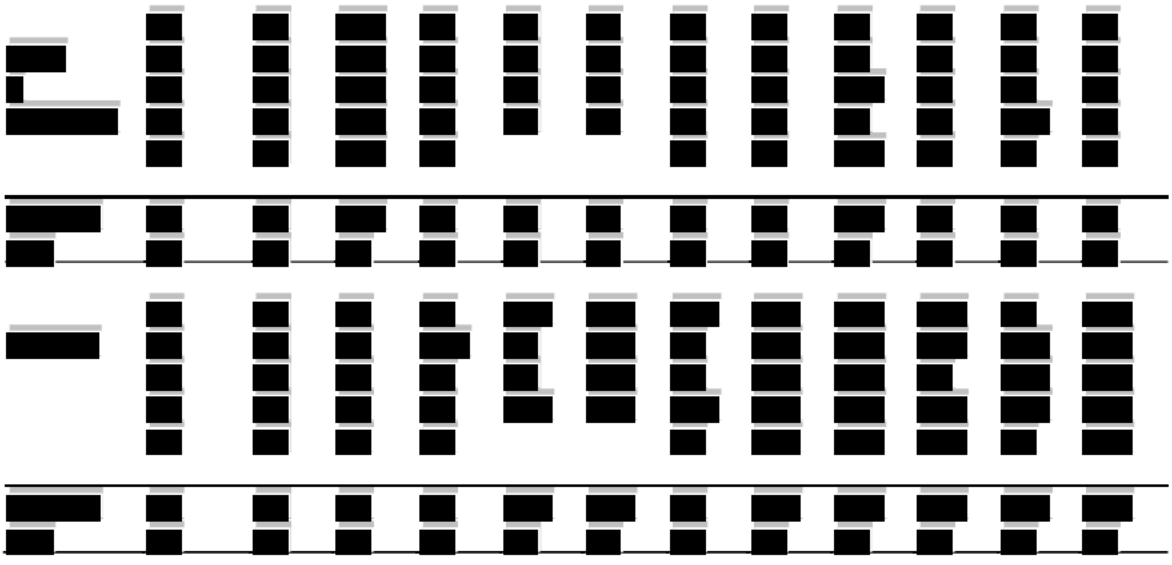
Table 1 Bioassays of 5 MYCOTAL batches on whitefly larvae: percentage of pupae alive, dead (+/- mycelium) and hatched per Petri dish.

Date	27.04.2004	06.05.2004	19.05.2004	26.05.2004	18.06.2004	30.06.2004

Batch	96MB423B		96M422		96M417		97M413		97M415		96M417	
Treatment	MYC	contr	MYC	contr	MYC	contr	MYC	contr	MYC	contr	MYC	contr



Date	27.04.2004		6.05.2004		19.05.2004		26.05.2004		18.06.2004		30.06.2004	
Batch	96MB423B		96M422		96M417		97M413		97M415		96M417	
Treatment	MYC	contr	MYC	contr	MYC	contr	MYC	contr	MYC	contr	MYC	contr



Precision

The development of the bioassay has been described by Girard (1989). She determined the effect of MYCOTAL in bioassays under different controlled conditions (spore density and relative humidity). Variance within treatments decreased with time of incubation and was lowest 8 - 12 days after inoculation.

To optimise the precision of the bioassay the inoculated whiteflies are incubated at a RH of 75 - 85% for 10 days.

Note RMS: Please applicant indicate more details about the visual differentiation of viable spores by their morphology.

New data 2016

For methods on contaminating micro-organisms please refer to B.5.1.7.

B.5.1.6 Methods for the determination of relevant impurities in the manufactured material

Information from the original DAR

A document was submitted that concerns a study on the production of destruxins by Mycotal (Skrobek, et al. (2005 submitted), submitted to Food and Chemical Toxicology); the study was part of the RAFBCA project. Tomato and cucumber plants received a foliar application of Mycotal at a dose of 108 spores/mL of spray (= 2 × 1014 spores/ha, i.e. 10 times the recommended dose). The tomato plants were treated 5 months after sowing, whereas the cucumber plants received a treatment 2 months after sowing. Mycotal was applied 3 times with an interval of 1 week. Fruits were sampled one day after the first application and one day after the third application (= 15 days after the first application). After harvesting, fruits were stored at -18ºC until analysis. For analysis, fruits were homogenised in TRIS buffer and pre-purified on Waters' C18 environmental cartridges using acetonitrile (leaves were not subjected to extraction and analysis). Extracts were re-dissolved in a methanol/acetonitrile-mixture. Additionally, tomato fruits were homogenised in a blender and extracted with a mixture of dichloromethane and ethyl acetate. To study the presence of metabolites in the product, both Mycotal and its unformulated spores were separately crushed in liquid nitrogen and extracted with dichloro-methane. The polar and non-polar fractions were subjected to analysis.

B.5.1.7 Methods to control the absence and to quantify (with appropriate limits of determination) the possible presence of any human and mammalian pathogen

Information from the original DAR

Identification of microbial impurities:

1. Aerobe mesophiles: Plate Count Agar, pour plates, 3 days at 30°C (Mossel & Tamminga, 1980)

2. Yeasts: Oxytetracycline Glucose Yeast extract Agar: pour plates, 3-5 days at 22°C (Dutch Food Legislation)
3. Coliformes: Violet Red Bile Lactose Agar: double layer, 20 hours at 30°C (ISO 4832)
4. Faecal Streptococci: Kanamycin Aesculin Azide Agar: brush plate, 3-5 days at 37°C (Mossel & Tamminga, 1980)
5. *Staphylococcus aureus*: Baird Parker Agar: brush plate, 48 hours at 37°C. Confirmation by use of coagulase test (Dutch Food Legislation)
6. *Salmonella* (Biochem work prescription): Non-selective accumulation: buffered peptone-water

Selective accumulation: Tetrathionate Brilliant green Gall-broth and Selenite broth, 18-24 hours at 42°C Confirm pure culturing Pure culturing: Xylose-Lysine-Deoxycholate-Agar and Bismuth-Sulfide-Agar, 18-24 hours at 37°C

Confirmation: short polyvalent O-serum, E-tube,Hoffman LaRoche

New data 2016

Microbial contaminants are to be analyzed with the standard methods summarized in the table below:

Table B.5.1.7-01: Determination methods for the identification of microbial contaminants

Contaminant	**Used Method**
E. coli	ISO 7251 or similar[1]
Salmonella sp.	ISO 6579
Staphylococcus aureus	ISO 6888-3
Aerobic bacteria	ISO 4833 or similar $<10^5$ CFU/g[1]

[1] For the description of the internal methods, see the information below

***E. coli* limit test**

A certain amount of sample is transferred to a tube containing a non-selective liquid enrichment medium. After incubation for 22 ± 2 hours at 30°C a certain amount is inoculated into a tube containing a liquid selective medium (Brilliant Green Bile Glucose Broth). This tube is incubated for 48 ± 2 hours at 30°C and checked for the forming of turbidity or gas. Both should be absent.

If turbidity or gas is formed a certain amount of this liquid is placed on a selective agar plate (Tryptone Bile Agar with X-Glucuronide) and streaked out. The plates are incubated at 44°C for 22 ± 2 hours. After incubation the plates are checked for the presence of blue colonies (*E. coli*).

Total aerobic plate count

A certain amount of sample or a series of dilutions of a sample is taken, potentially after pasteurization, and inoculated on Plate Count Agar. After incubation (37 °C) the amount of formed colonies are counted and the total colony forming units per amount of sample is calculated.

The used internal methods are performed by a laboratory, accredited under ISO/IEC 17025 by the Dutch Accreditation Council RvA. This means that results have been obtained through the application of (inter)national standards or in-house methods that have been fully validated.

Analysis of *Listeria monocytogenes* is not required, since hygienic indicators constantly demonstrate acceptably low levels of contamination. For more information, please refer to the confidential information in Volume 4.

B.5.1.8 Methods to determine storage stability, shelf-life of the micro-organism, if appropriate

Information from the original DAR

The amount of biologically active units is defined as the fraction of viable spores (germination test) multiplied by the amount of spores per gram (spore content). The amount of non-germinated and germinated spores after 18 hours at room temperature is determined in the germination test. The amount of spores is determined with a counting chamber according to the method of Bürker-Türk.

New data 2016

Previously submitted information is considered to be acceptable to cover current requirements, with the exception that new method validation was provided.

For detailed information, please refer to Volume 4.

[redacted]

[redacted]

B.5.2 Methods to determine and quantify residues (viable or non-viable) of the active micro-organism

B.5.2.1 The active micro-organism(s) on and/or in crop, in foodstuffs and feeding stuffs, in animal and human body tissues and fluids, in soil, in water (including drinking water, ground water and surface water) and in air where relevant (i.e. viable residues)

Information from the original DAR

See doc B.7.1 MA. Residues of the active micro-organism are determined by plating samples onto malt agar extract or selective medium (Rose bengal chloramphenicol agar). Colonies can be identified by morphological identification methods (see addendum 2009 B.5.1.5 MA).

New data 2016

Previously submitted information is considered to be acceptable to cover current requirements.

B.5.2.2 Relevant metabolites (especially toxins) on and/or in crop, in foodstuffs and feeding stuffs, in animal and human body tissues and fluids, in soil, in water (including drinking water, ground water and surface water) and in air where relevant (i.e. non -viable residues)

Information from the original DAR

See doc B.7.1 MA.

A method to determine non-viable residues is not necessary.

New data 2016

Previously submitted information is considered to be acceptable to cover current requirements.

B.5.3 References relied on

Data point	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Data protection claimed Y/N	Owner
KMA 4.2/07	Registration department Koppert B.V.	2016	STATEMENT Koppert, not stated Koppert B.V., Berkel en Rodenrijs, NL GLP/GEP: no Published: no	yes	KBS
	Koppert Beheer BV	2005	Identity and content of impurities and contaminating micro-organisms. Koppert Beheer BV, Department R&D Microbials and Regulatory affairs, P.O. Box 155, 2650 AD Berkel en Rodenrijs, The Netherands Koppert Beheer BV - - unpublished statement	Y	KBS
	Koppert Beheer BV	2003b	Method of quality control of *Verticillium lecanii* strain Ve6: viable spore content. Koppert Beheer BV, Department R&D Microbials and Regulatory affairs, P.O. Box 155, 2650 AD Berkel en Rodenrijs, The Netherands Koppert Beheer BV - - unpublished statement	Y	KBS
	Koppert Beheer BV	2001	Method of quality control of *Verticillium lecanii* strain Ve6: Qualitative / Laboratory bio-essay. Koppert Beheer BV, Department R&D Microbials and Regulatory affairs, P.O. Box 155, 2650 AD Berkel en Rodenrijs, The Netherands Koppert Beheer BV - - unpublished statement	Y	KBS

Data point	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Data protection claimed Y/N	Owner
	Koppert Beheer B.V.	2005	Information on the production of metabolites (especially toxins) by *Verticillium lecanii*. Koppert Beheer B.V., Department R&D Microbials and Regulatory affairs, P.O. Box 155, 2650 AD Berkel en Rodenrijs, The Netherands; Koppert Beheer B.V. - - unpublished statement	Y	KBS
	Koppert Beheer B.V.	2004f	Genetic stability of *Verticillium lecanii* strain Ve6 and factors affecting it Koppert Beheer B.V., Department R&D Microbials and Regulatory affairs, P.O. Box 155, 2650 AD Berkel en Rodenrijs, The Netherands Koppert Beheer BV - - unpublished statement	Y	KBS
	Koppert B.V.	2006	Material Safety Data Sheet *Lecanicillium muscarium* strain Ve6, version 1.1 Koppert B.V., Department R&D Microbials and Regulatory affairs, P.O. Box 155, 2650 AD Berkel en Rodenrijs, The Netherlands Koppert BV - - unpublished statement	N	KBS
	Koppert Beheer B.V.	2005	Reproducibility of quality control tests of *Verticillium lecanii*. Koppert Beheer B.V., Department R&D Microbials and Regulatory affairs, P.O. Box 155, 2650 AD Berkel en Rodenrijs, The Netherlands. Koppert Beheer B.V. - Not GLP Unpublished	Y	KBS